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# A New Continuous Epitope of Hepatitis A Virus

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A new continuous epitope of hepatitis A virus (HAV) was defined in the VP3 protein. Convalescent sera recognised the synthetic peptide 3110–3121 (FWRGDLVFDFQV). The replacement of the arginine, glycine, or aspartic acid at positions 112, 113, or 114, respectively by other amino-acids induced the loss of synthetic peptide recognition by human convalescent sera, thereby confirming the presence of an epitope in the original VP3(110-121) sequence. Shorter VP3 peptides such as VP3(110-119), VP3(110-117), and VP3(110-116) and a tandem repeat of VP3(111-116) failed to react with convalescent sera, indicating the importance of the entire peptide in the epitope structure. The maximum inhibition of human convalescent binding to HAV by the VP3(110-121) peptide was around 60%, and 50% inhibition was achieved at a peptide concentration of 2.3–2.4 µg/ml. Antibodies generated by this peptide bound to intact HAV and neutralised its infectivity. Antipeptide antibodies inhibited convalescent serum binding to HAV. Monoclonal antibodies H7C27 and MAK-4E7 inhibited completely binding of the antipeptide antibodies to HAV. ***J. Med. Virol. 54:95–102, 1998.***


**KEY WORDS:** synthetic peptides; structure; monoclonal antibodies

## INTRODUCTION

Hepatitis A virus (HAV) is an important hepatotropic virus classified as the type species of the genus *Hepatovirus* within the *Picornaviridae* family [Murphy et al., 1995]. The virion capsid is composed of structural proteins: VP1, VP2, VP3, and, possibly, VP4 [Lemon and Robertson, 1993]. There are only a limited number of antigenic sites. The immunodominant site, composed of closely clustered epitopes, is defined by two major groups of escape mutants that include residues 70 and 74 of VP3 and residues 102, 171, and 176 of VP1 [Nainan et al., 1992; Ping and Lemon, 1992; Lemon and Robertson, 1993]. There is another apparently distinct antigenic site represented by mutants at residue 221 of VP1 and an additional and still undefined third antigenic site because a monoclonal antibody (MAb) effectively neutralises all escape mutants so far isolated [Ping and Lemon, 1992; Lemon and Robertson, 1993]. Twenty of 22 murine MAbs are directed towards the immunodominant site, and H7C27 and MAK-4E7 are directed against the other two antigenic sites, respectively.

Information on the antigenic structure of HAV has been obtained after the isolation of escape mutants resistant to MAbs because the major neutralisation epitopes of HAV appear to be discontinuous [Stapleton and Lemon, 1987]. However, other widely used techniques for the characterisation of antigenic sites are based on the binding of mono- and polyclonal antibodies to synthetic peptides representing parts of the primary sequence of capsid proteins.

In the present study, three synthetic peptides were selected for the search of antigenic sites of HAV: VP1(11-25) (TVSTEQNVPDPQVGI) [Emini et al., 1985], the surface-exposed VP2(96-107) (GLLRYHTYARFG) [Robertson et al., 1989], and the hydrophilic VP3(110-121) (FWRGDLVFDFQV) [Wheeler et al., 1986], which includes the tetrapeptide RGDL and is also included in neutralisation site A of foot-and-mouth-disease virus [Verdaguer et al., 1995].

## MATERIALS AND METHODS

### Viruses and Cells

FRhK-4 cell cultures were used to propagate and assay the cytopathogenic HM-175 (courtesy of T. Cromeans, Centers for Disease Control, Atlanta, GA) strain of HAV [Cromeans et al., 1987]. Viral enumera-

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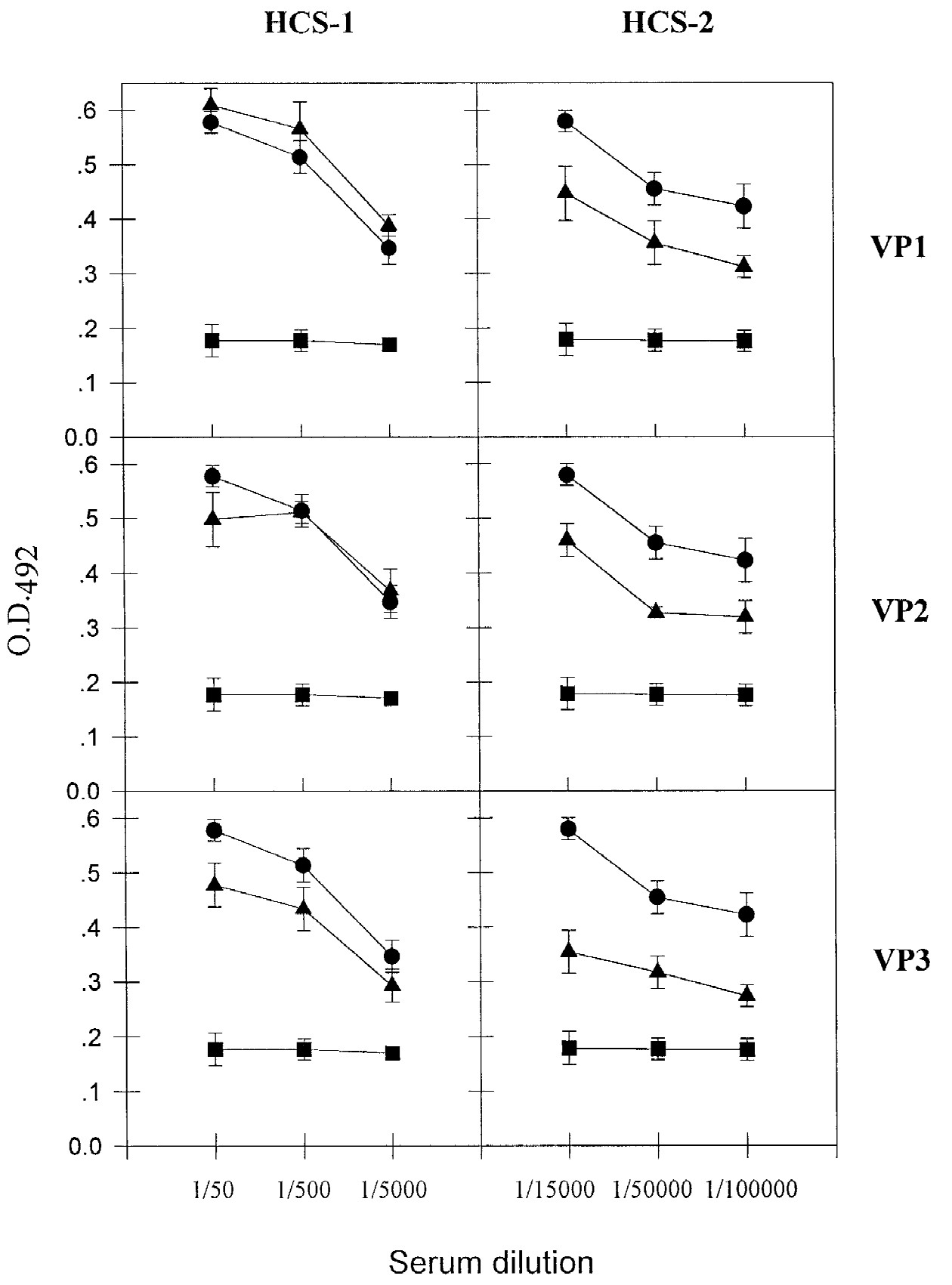


Fig. 1. Recognition of HAV-related synthetic peptides by human convalescent sera by competitive ELISA. Human sera (HCS-1 and HCS-2) were preincubated with BSA (circle) or synthetic peptides (triangle) prior to reaction with HAV. FRhK-4 cell lysates (square) were used as negative control for HAV recognition.

TABLE I. Recognition of HAV VP3 Peptides by a Human Convalescent Serum[a]

| VP3 Sequence | HAV recognition after BSA serum adsorption | HAV recognition after peptide serum adsorption | FRhk-4 cell recognition after BSA serum adsorption | Peptide recognition |
|---|---|---|---|---|
| FWRGDLVFDFQV | 0.504 ± 0.001 | 0.274 ± 0.012 | 0.234 ± 0.003 | + |
| FW**A**GDLVFDFQV | 0.504 ± 0.001 | 0.452 ± 0.020 | 0.234 ± 0.003 | − |
| FWR**A**DLVFDFQV | 0.504 ± 0.001 | 0.452 ± 0.016 | 0.234 ± 0.003 | − |
| FWRG**A**LVFDFQV | 0.504 ± 0.001 | 0.442 ± 0.026 | 0.234 ± 0.003 | − |
| FW**S**GDLVFDFQV | 0.504 ± 0.001 | 0.484 ± 0.018 | 0.234 ± 0.003 | − |
| FW**K**GDLVFDFQV | 0.504 ± 0.001 | 0.542 ± 0.024 | 0.234 ± 0.003 | − |
| FWRG**E**LVFDFQV | 0.504 ± 0.001 | 0.377 ± 0.017 | 0.234 ± 0.003 | + |
| FWRG**K**LVFDFQV | 0.504 ± 0.001 | 0.374 ± 0.015 | 0.234 ± 0.003 | + |
| FW**E**GDLVFDFQV | 0.504 ± 0.001 | 0.457 ± 0.018 | 0.234 ± 0.003 | − |
| FWRG**R**LVFDFQV | 0.504 ± 0.001 | 0.422 ± 0.012 | 0.234 ± 0.003 | + |
| FW**D**GDLVFDFQV | 0.504 ± 0.001 | 0.482 ± 0.020 | 0.234 ± 0.003 | − |
| FWR**E**DLVFDFQV | 0.504 ± 0.001 | 0.528 ± 0.029 | 0.234 ± 0.003 | − |
| FWR**K**DLVFDFQV | 0.504 ± 0.001 | 0.472 ± 0.022 | 0.234 ± 0.003 | − |
| FW**AA**DLVFDFQV | 0.504 ± 0.001 | 0.397 ± 0.016 | 0.234 ± 0.003 | + |
| FWR**AA**LVFDFQV | 0.504 ± 0.001 | 0.472 ± 0.016 | 0.234 ± 0.003 | − |
| FW**A**G**A**LVFDFQV | 0.504 ± 0.001 | 0.446 ± 0.018 | 0.234 ± 0.003 | − |
| FWRGDLVFDF-- | 0.504 ± 0.001 | 0.446 ± 0.013 | 0.234 ± 0.003 | − |
| FWRGDLVF---- | 0.504 ± 0.001 | 0.471 ± 0.017 | 0.234 ± 0.003 | − |
| FWRGDLV----- | 0.504 ± 0.001 | 0.495 ± 0.018 | 0.234 ± 0.003 | − |
| WRGDLV**WRGDLV** | 0.504 ± 0.001 | 0.516 ± 0.026 | 0.234 ± 0.003 | − |

[a]Numbers (mean ± SD of three independent experiments with all peptides) refer to recognition by human convalescent serum (HCS-2, diluted 1/50,000) of HAV or FRhK-4 cell lysates by a sandwich ELISA ($O.D._{492}$). Bold residues correspond to substitutions. Hyphens correspond to deleted residues. The last sequence corresponds to a tandem repeat of amino acids 111–116.

tions were carried out by calculating the most probable number of cytopathogenic units per millilitre (MPNCU/ml) by infecting cell monolayers grown in 96-well microtitre plates [Pintó et al., 1994]. Sixteen wells were infected for each dilution, and 20 μl of inoculum were added to each well. Data were processed with a MPN computer programme [Hurley and Roscoe, 1983].

### HAV Human Convalescent Sera

Two convalescent sera (HCS-1 and HCS-2) were generously provided by Dr R. Lluna, Hospital Militar, Barcelona, and characterised for their ability to react with HAV in a sandwich enzyme-linked immunoabsorbent assay (ELISA). This ELISA consisted of an HAV capture by MAb 33Z/37/39 and detection through reaction with the convalescent sera. Mock-infected FRhK-4 cell lysates were used as negative controls. HCS-1 and HCS-2 were able to bind to HAV at dilutions of 1/10,000 and 1/1,000,000, respectively.

### HAV Monoclonal Antibodies

The following MAbs against HAV have been used in competitive studies of HAV recognition: K3-4C8, purchased from Commonwealth Serum Laboratories (Victoria, Australia); B5B3, generously provided by Dr. B. Ferns, University College London Medical School (London, UK); H7C27, generously provided by Dr. R. Decker (Abbot Laboratories, North Chicago, IL); and MAK-4E7, generously provided by Dr. B. Flehmig, University of Tübingen (Tübingen, Germany). All MAbs were properly diluted to achieve an immunoglobulin concentration of 1 mg/ml. The MAb 33Z/37/39, generously provided by Dr. Z.-M. Yun, Institute of Virology (Beijing), was used as capture antibody in solid-phase immunoassays [Guo et al., 1992].

### Peptide Synthesis and Induction of Antipeptide Antibodies

The synthesis of the linear peptides was accomplished by the continuous-flow Fmoc-polyamide solid-phase method [Stewart, 1983]. Six-week-old female Swiss mice were used to obtain ascitic antibodies after immunisation with 250 μg of the synthetic peptides entrapped in liposomes [Haro et al., 1995] by employing Freund's complete adjuvant (FCA) as an enhancer. Ascites generated by inoculation of phosphate buffered saline (PBS) were used as negative controls, and ascites generated by an inoculation of 30 ng of intact HAV particles were used as positive controls. For the VP3(110-121) peptide, antisera also were obtained in rabbits after immunisation with 1,500 μg of the entrapped peptide.

### Recognition of HAV Peptides

A competitive test was employed to assay the recognition of the synthetic peptides by human convalescent sera HCS-1 and HCS-2. The sera were preincubated for 2 hours at 37°C with either 10 μg of free peptide or bovine serum albumin (BSA) in an equimolecular ratio per millilitre of sera and tested for HAV recognition in a sandwich ELISA. HAV or FRhK-4 cell lysates were captured through MAb 33Z/37/39 and detected by HCS-1 or HCS-2. A statistically significant ($P < 0.05$, analysis of variance [ANOVA]) decrease in HAV recognition by the convalescent sera preincubated with HAV-related peptides with regard to the same sera

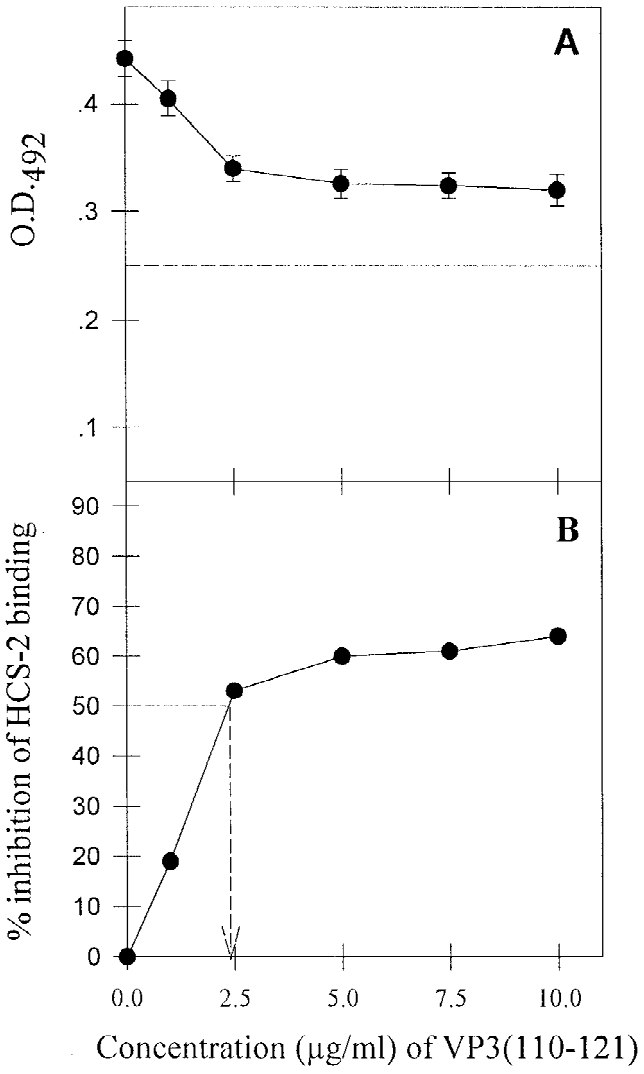


Fig. 2. VP3(110-121) recognition by a human convalescent serum (HCS-2) expressed as the decrease of HAV recognition after incubation of the serum with the peptide. **A:** ELISA readings of HAV recognition (dashed line represents the ELISA reading for FRhK-4 cell lysates). **B:** Percent of inhibition of HAV recognition (dashed arrow represents the peptide concentration for a 50% inhibition).

preincubated with BSA was indicative of peptide recognition. The amount of peptide required for a 50% inhibition of HCS-2, diluted to 1/50,000, binding to HAV was calculated.

Several analogues of the VP3 peptide also were synthesised and assayed for recognition with HCS-2 diluted 1/50,000.

Peptide recognition by antipeptide antibodies was also assayed by a direct ELISA, in which plates were coated with each peptide and then reacted with ascitic fluids generated against each peptide.

### HAV Recognition by Antipeptide Antibodies

HAV recognition was tested by a competitive ELISA, consisting of a preincubation of ascitic fluids or sera with intact HAV virus ($5 \times 10^5$ MPNCU/ml of sample) for 2 hours at 37°C, before addition to the immobilised peptides. Supernatants from mock-infected FRhK-4 lysates were incubated with ascites and used as negative controls. A statistically significant ($P < 0.05$, ANOVA) decrease in peptide recognition by the antipeptide ascites preincubated with HAV with regard to the same ascites preincubated with mock-infected cell lysates indicated virus recognition.

TABLE II. Anti-HAV Responses After Immunisation of Mice With Synthetic Peptides[a]

| Peptide | HAV recognition[b] | Neutralisation[c] |
|---|---|---|
| VP1(11-25) | | |
| 1 (●)[d] | 1/20 | 5.48 |
| 2 (■) | — | 5.44 |
| 3 (▲) | — | 0 |
| VP2(96-107) | | |
| 1 (●) | — | 0 |
| 2 (■) | — | 0 |
| 3 (▲) | 1/4 | 5.35 |
| VP3(110-121) | | |
| 1 (●) | 1/20 | 5.20 |
| 2 (■) | 1/20 | 5.51 |
| 3 (▲) | — | 5.49 |
| 4 (♦) | — | 5.30 |

[a]Six mice were used for each peptide. Only data from mouse ascites showing antipeptide recognition are depicted.
[b]Dilution positive for HAV recognition. Dash indicates no recognition at a 1:4 dilution.
[c]Neutralisation index: $\log_{10}$ reduction of initial virus titer ($3.5 \times 10^5$ MPNCU/ml). Ascites were assayed for HAV neutralisation diluted to 1:4.
[d]Depicted symbols are the same as those in Figure 3.

### Competitive Inhibition of Virus Recognition by the Different Antibodies

The capacity of peptide-induced antibodies to inhibit the binding of a convalescent serum to intact virions was tested by a competitive ELISA. Viruses or mock-infected cell lysates were captured by MAb 33Z/37/39 and incubated with either antipeptide antibodies or antibodies not related to HAV (ascitic fluid induced with PBS) prior to their detection by the human convalescent serum. Competing antibodies were assayed at dilutions yielding maximum HAV recognition, and human convalescent serum HCS-2 was used at a 1/50,000 dilution. As a negative control, FRhK-4 cell lysates were incubated with nonrelated antibodies. The inhibition of human convalescent serum binding was considered to be 100% when optical densities equalled that of the negative control.

Another competitive ELISA was employed to evaluate the inhibition of the rabbit anti-VP3(110-121) serum binding to HAV by different MAbs. These MAbs, from stock solutions containing 1 mg/ml of immunoglobulins, were titrated previously by competitive inhibition of human convalescent serum HCS-2 binding to HAV. Viruses or FRhK-4 cells were immobilised with the 33Z/37/39 MAb and incubated with different dilutions of the competing MAbs before detection with the human convalescent serum at a 1/50,000 dilution. Ascitic fluid induced with PBS was used as a reference

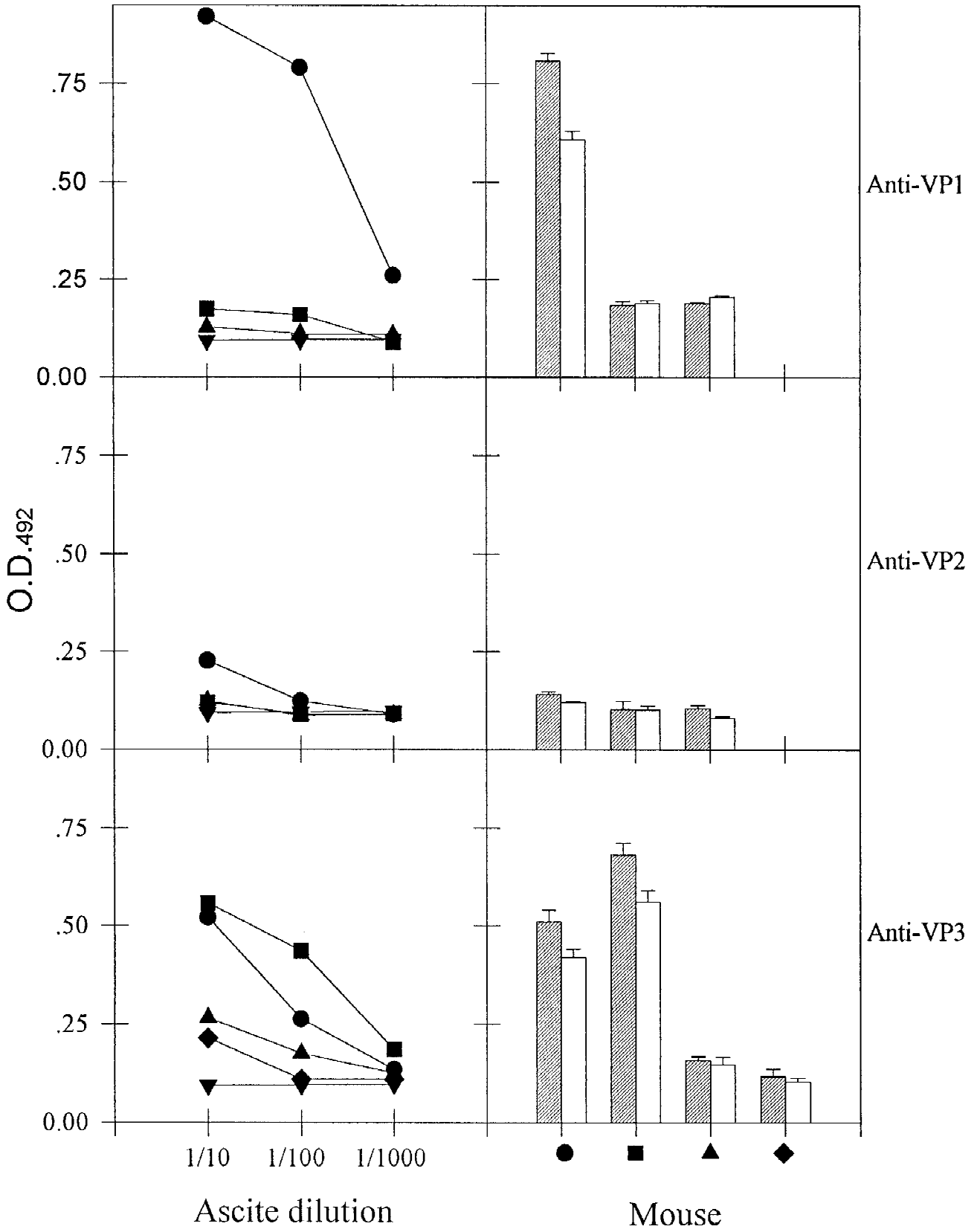


Fig. 3. Synthetic peptide recognition **(left)** and HAV recognition **(right)** by antipeptide ascitic fluids from different animals. Peptide recognition by ascites from individual mouse (circle, square, triangle up, and diamond; see Table II) was assayed by direct ELISA. Nonimmune ascites (triangle down) were used as negative controls. For HAV recognition, the same antipeptide ascites were preincubated with either FRhK-4 cell lysates (filled bar) or HAV (empty bar) prior to being added to peptides. A separate batch of mice was employed for each peptide.

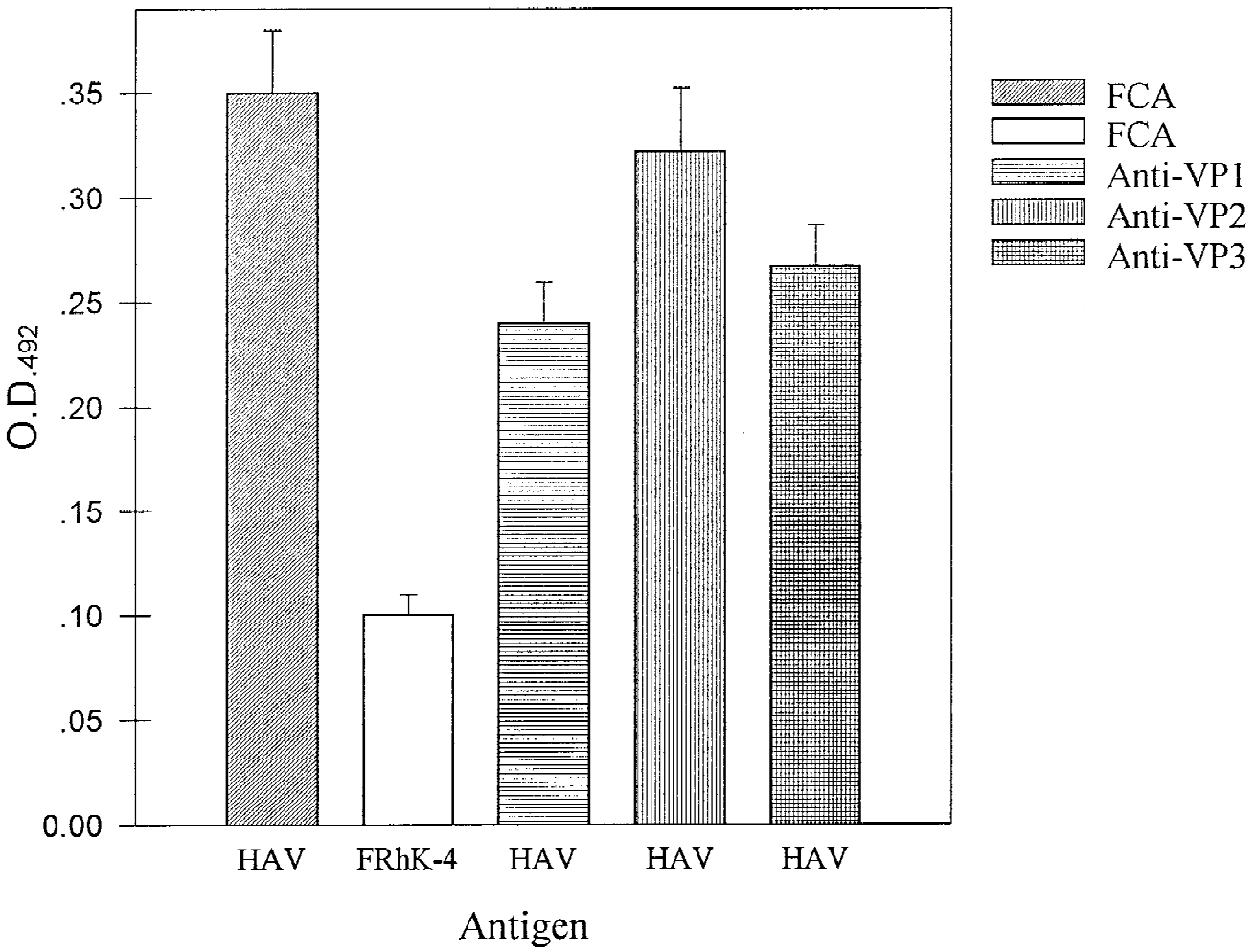


Fig. 4. Competitive inhibition of human convalescent antibody binding to HAV by antisynthetic peptide antibodies. HAV or FRhK-4 cell lysates (negative control) were incubated previously with ascites containing antibodies vs. nonrelated antigen (FCA), VP1 peptide (Anti-VP1), VP2 peptide (Anti-VP2), or VP3 peptide (Anti-VP3) and then detected by a human convalescent serum.

noncompetitive control, and FRhK-4 cell lysates incubated with this HAV nonrelated ascitic fluid were used as negative controls. The MAb dilutions yielding around 40% inhibition were then used to compete with the rabbit anti-VP3(110-121) serum. Immobilised viruses or cell lysates were incubated with the competing MAbs or nonrelated antibodies and detected with the rabbit anti-VP3 serum at a dilution yielding maximum HAV recognition. FRhK-4 cell lysates were incubated with either nonrelated antibodies or each MAb and used as negative controls. The inhibition of anti-VP3 serum binding was considered to be 100% when optical densities equalled that of the corresponding negative control.

### HAV Neutralisation

Ascitic fluids and sera showing peptide recognition were assayed for their capacity to neutralise the infectivity of suspensions ($3.5 \times 10^5$ MPNCU/ml) of HAV, strain HM-175, for 3 hours at 37°C, as described elsewhere [Haro et al., 1995]. The neutralisation index was calculated as the $\log_{10} (N_0 - N_f)$, with $N_0$ as the initial virus titre and $N_f$ as the final virus titre after neutralisation.

All experimental procedures were carried out at least in triplicate.

## RESULTS AND DISCUSSION

HAV synthetic peptides were assayed for recognition by human convalescent sera. Among the three peptides tested, the VP3(110-121) was recognised by both high-titre (HCS-2) and low-titre (HCS-1) human anti-HAV sera (Fig. 1), whereas VP1(11-25) and VP2(96-107) were only recognised by the high-titre convalescent serum. This result led us to investigate the effect of selected aminoacid replacements and deletions in the sequence of the VP3 peptide on recognition by human convalescent sera. Because the RGD motif has been involved directly in the interaction of antibodies with the major antigenic loop of foot-and-mouth-disease virus [Verdaguer et al., 1995], amino acid replacements in this specific sequence were evaluated. The replace-

ment of the arginine, glycine, or aspartic acid at positions 112, 113, or 114, respectively, by other amino acids induced the loss of synthetic peptide recognition by HCS-2 (Table I), thus confirming the presence of an epitope in the original VP3(110-121) sequence. Only three changes in a single amino acid (RGE, RGK, and RGR) and a two-amino-acid change (AAD) did not result in the loss of recognition by HCS-2. Moreover, not only the RGD region is implicated in the epitope structure because shorter VP3 peptides including this motif, such as VP3(110-119), VP3(110-117), and VP3(110-116), and a tandem repeat of VP3(111-116) failed to react with HCS-2. The inhibition of HCS-2 binding to HAV by different concentrations of the VP3(110-121) peptide was investigated. Maximum inhibition (around 60%) was achieved at concentrations higher than 2.5 μg/ml (Fig. 2). The amount required for a 50% inhibition was determined from the linear portion of the inhibition curve and was 2.3–2.4 μg/ml.

The immune responses elicited by the synthetic peptides were investigated. The VP3(110-121) peptide showed a higher immunogenicity than did peptides VP1(11-25) and VP2(96-107) in terms of number of responding animals and induction of antibodies showing viral recognition and neutralisation (Table II and Fig. 3).

Assays of competitive inhibition of human convalescent antibody binding to HAV by antisynthetic peptide antibodies were undertaken. HCS-2 binding to HAV was significantly ($P < 0.05$) inhibited by preincubation with anti-VP1(11-25) (44% inhibition) and anti-VP3(110-121) (35% inhibition) antibodies but not by VP2(96-107) (Fig. 4). Thus, the virion epitopes containing VP1(11-25) and VP3(110-121) are recognisable by the human immune system.

To characterise the new VP3 epitope, competitive assays for HAV recognition were carried out with MAbs associated with the antigenic sites and a rabbit anti-VP3(110-121) antibody. HAV MAbs were titrated previously by assaying their ability to inhibit competitively the binding of HCS-2 to HAV (Fig. 5). MAbs K3-4C8, B5B3, H7C27, and MAK-4E7 (stock solutions of 1 mg/ml of total antibody concentration) diluted to 1/10,000 induced 48%, 44%, 37%, and 41% inhibition of HCS-2 binding to HAV, respectively. This MAb concentration was then used in the assays of competition with the rabbit anti-VP3(110-121) serum. All these MAbs induced a significant ($P < 0.05$) inhibition of anti-VP3(110-121) binding to HAV (Fig. 6), with inhibitions of 50% with K3-4C8 antibodies, 76% with B5B3 antibodies, and 100% with both H7C27 and MAK-4E7 antibodies. Although the VP3(110-121) peptide was not recognised by any of the MAbs that are characteristic of the HAV antigenic sites (data not shown), the aforementioned different levels of competition suggest that the VP3 epitope is located closer to the antigenic sites defined by the MAbs H7C27 and MAK-4E7 than to the immunodominant site. No actual crystallographic data on the structure of HAV exist, but two models have been developed based on the structure of other picor-

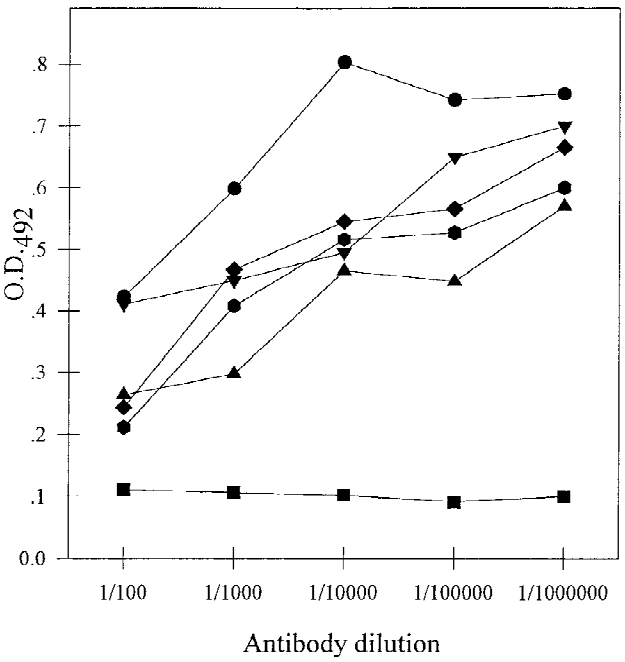


Fig. 5. Competitive inhibition of human convalescent antibody binding to HAV by different monoclonal antibodies. HAV was incubated previously with nonrelated ascites (circle), K3-4C8 ascites (triangle up), B5B3 ascites (triangle down), H7C27 ascites (diamond), or MAK-4E7 ascites (hexagon) and then detected with a human convalescent serum. FRhK-4 cell lysates were incubated with HAV nonrelated ascites (square) and used as negative controls.

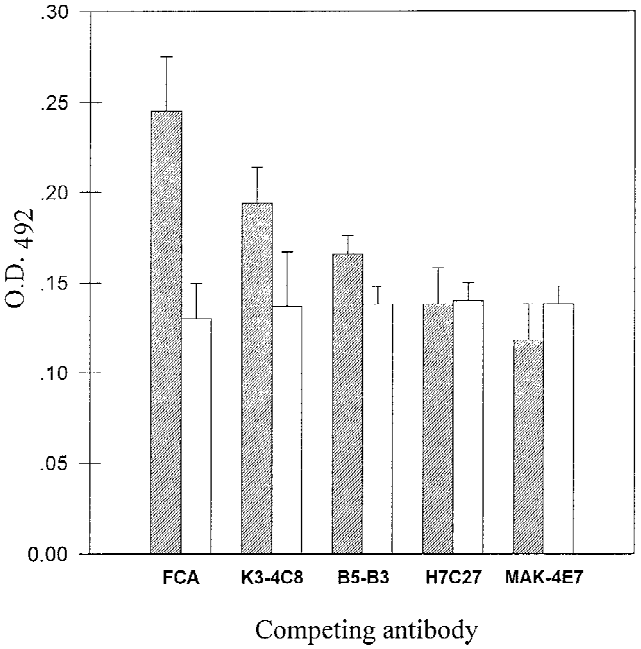


Fig. 6. Competitive inhibition of anti-VP3(110-121) antibody binding to HAV by different monoclonal antibodies. HAV (filled bar) or FRhK-4 cell lysates (empty bar) were incubated with antibodies unrelated to HAV (FCA) or with different MAbs prior to being detected with the anti-VP3 serum.

navirus [Luo et al., 1988; M. Luo and L. Zhou, personal communication]. In both models, the phenylalanine 3110 of the VP3 sequence is located closely to the lysine 1221 of VP1, with the latter being the residue identified by the escape mutants to the H7C27 MAb. The relationship between the VP3 sequence and the antigenic site defined by the MAb MAK-4E7 remains unclear because no escape mutant has been described. However, according to both models, the VP3 sequence is not exposed on the capsid surface, with only some residues such as 3110, 3115, 3118, and 3119 being located in the deepest virus surface. In spite of this, the VP3 epitope should be accessible to the antibodies because experimental results have shown that the antibodies induced by this sequence interact with the intact virion and that the sequence is recognised by different human convalescent sera. It should be noted that the latest model of HAV structure is the result of refinements made to the first model [Luo et al., 1988] to locate the amino acids identified by escape mutants on the virion surface and that, in this second model, the residues of the VP3 and VP1 proteins identified in the immunodominant antigenic site are still spatially too far one from another to be included in a single immunoglobulin binding site [Nainan et al., 1992]. To resolve this question, it has been suggested that mutations in one residue could affect the immunoglobulin binding to the other, although they are very ditant from one another [Nainan et al., 1992]. However, the very exposed VP2(96-107) sequence [Luo et al., 1988; Robertson et al., 1989] failed to induce an immune response comparable to that of VP3(110-121). In any case, all these paradigms cannot be answered before the resolution of the actual HAV capsid structure.

The continuous nature of the VP3(110-121) epitope was confirmed by Western blot analysis of purified HAV. The denatured VP3 protein reacted with both the anti-VP3(110-121) peptide antibody and an anti-VP3(31-47) peptide antibody (gift of D.V. Sangar) used as positive control serum (data not shown).

Most of the antigenic determinants of proteins are discontinuous. However, it is nevertheless possible to mimic such epitopes by means of linear, synthetic peptides. When such peptides are found to cross-react with antiprotein antibodies or when they are able to induce antibodies that cross-react with the parent protein, the peptides are called continuous epitopes [van Regenmortel, 1993]. A new linear epitope of HAV has been described by using this approach.

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